

Melatonin Metabolism: Neural Regulation of Pineal Serotonin: Acetyl Coenzyme A *N*-Acetyltransferase Activity

(rat/diurnal rhythm/melatonin/*N*-acetylserotonin/organ culture/circadian enzyme regulation)

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ABSTRACT There is a diurnal rhythm in the activity of serotonin *N*-acetyltransferase in the rat pineal gland. In the normal rat, the nocturnal enzyme activities are 15- to 30-fold greater than are day time activities. This rhythm is abolished by decentralization or removal of the superior cervical ganglia, procedures that interrupt the only source of central neural input to the pineal gland. This effect of superior cervical sympathectomy on the *N*-acetyltransferase rhythm cannot be attributed to changes occurring in the denervated pineal parenchymal cells. When chronically denervated glands are placed in organ culture with norepinephrine, the neurotransmitter normally located in sympathetic terminals in the gland, *N*-acetyltransferase activity increases 10- to 20-fold. These data indicate that superior cervical sympathectomy abolishes the *N*-acetyltransferase rhythm by elimination of the input of central signals to the gland. These signals appear to regulate the *N*-acetyltransferase rhythm in the normal rat by regulation of the release of norepinephrine from the sympathetic terminals within the pineal gland.

N-acetyltransferase transfers an acetyl group from acetyl coenzyme A to the amino group of serotonin (5-hydroxytryptamine) to form *N*-acetylserotonin (5-hydroxy-*N*-acetyltryptamine) (1). *N*-acetylserotonin is the precursor of melatonin (5-methoxy-*N*-acetyltryptamine) (2), the putative pineal hormone, which is formed primarily in the pineal gland (3). In the rat pineal gland there is a 24-hr rhythm in *N*-acetyltransferase activity (4). During the light period of a normal lighting schedule, *N*-acetyltransferase activity is low. After the onset of darkness, the activity of this enzyme increases to values that are 15- to 30-fold greater than those that occur during the light period (4). The rhythm is maintained after animals are blinded or placed in constant darkness (4, 5). Exposure to constant light suppresses the rhythm, and results in continually low activity of pineal *N*-acetyltransferase (4). These characteristics indicate that the *N*-acetyltransferase rhythm is like many other 24-hr rhythms, which are driven by an endogenous mechanism in the central nervous system and can be suppressed or shifted by environmental lighting (6).

The only known pathway of central input to the pineal gland is via sympathetic nerves from the superior cervical ganglia (SCG) (7). Evidence indicates that the release of norepinephrine from sympathetic terminals regulates pineal *N*-acetyltransferase. First, a 10- to 20-fold increase in *N*-acetyltransferase results when norepinephrine is added to pineal organ cultures (8). Second, pineal norepinephrine is contained entirely in nerve processes (9-11). Serotonin, which

is also located in these processes, and tryptamine cannot induce *N*-acetyltransferase in cultured pineal glands (4, 12). Third, there is a 24-hr rhythm in pineal norepinephrine content that is abolished by decentralization of the SCG (13).

To test directly whether the SCG mediate neural control of the *N*-acetyltransferase rhythm, we examined the effects of removal or decentralization of them. Using organ cultures of pineal glands, we also determined whether chronic denervation affects the ability of pineal cells to respond to norepinephrine, as indicated by an increase of *N*-acetyltransferase activity and an increase in the amount of [³H]-melatonin formed from [³H]tryptophan.

MATERIALS AND METHODS

Control and surgically-prepared male Sprague-Dawley rats (150-180 g) were obtained commercially (Zivic-Miller Laboratories, Inc., Alison Park, Pa.). Three groups of operated animals were obtained. One group was subjected to bilateral removal of the SCG, a second group to bilateral decentralization of the SCG, and a third group to a sham operation in which a surgical incision was made in the neck similar to that made for ganglionectomy or decentralization. All of the animals subjected to superior cervical ganglionectomy or decentralization exhibited a bilateral ptosis. The animals were delivered within 48 hr after surgery and groups of four were then placed in transparent plastic cages (15 × 25 × 41 cm) in National Institutes of Health animal facilities. The animals were maintained for 21 days on a schedule of diurnal lighting in which the room lights were on for 14 hr (from 7 a.m. to 9 p.m.) and off for 10 hr. The light was provided by incandescent bulbs and had an intensity in the cages of 100-350 lm/meter². Food (Purina Lab Chow) and water were available at all times.

At the conclusion of the experiment, the animals were stunned by a blow on the head and then decapitated. Those killed at night were exposed to a dim white light for less than 1 min. Pineal glands were rapidly removed and, unless otherwise noted, were stored for less than 5 min in physiological saline at room temperatures before being assayed for *N*-acetyltransferase activity or placed in organ-culture vessels.

N-acetyltransferase activity was determined by measurement of the [¹⁴C]*N*-acetylserotonin and [¹⁴C]melatonin formed by the homogenate of an individual pineal gland incubated for 10 min at 37°C in 20 μl of 0.1 *M* sodium phosphate buffer (pH 6.8) containing 1 mM [¹⁴C]serotonin (28 Ci/mol) and 2 mM acetyl coenzyme A. Radiolabeled *N*-acetylserotonin and melatonin were isolated by thin-layer chromatography

Abbreviation: SCG, superior cervical ganglia.

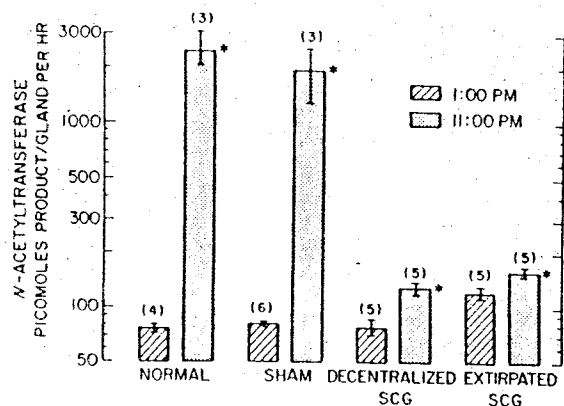


FIG. 1. Pineal *N*-acetyltransferase activity. Numbers in parentheses indicate the number of animals in each group. Superior cervical ganglia (SCG) were removed or decentralized 3 weeks before pineal glands were obtained.

* Night value was greater than day value $P < 0.05$, as indicated by 2-tailed Student's *t* test (9).

and measured (4). Less than 5% of the [14 C]*N*-acetylserotonin is converted to [14 C]melatonin. The term "picomoles of product" in the legends describes the total radiolabeled *N*-acetylserotonin and melatonin formed. Based on the results of studies in which preparations of pineal glands obtained during the day, when enzyme activity is low, are added to preparations obtained during the night, when enzyme activity is high, it appears that the apparent changes in enzyme activity are not due to the presence in the enzyme assay of endogenous inhibitors, activators, precursors, or products.

Intact rat pineal glands were cultured in chemically defined media (BGJ^b) (5). Incubations began about 9 a.m. [3 H]Tryptophan (0.2 mM, 25 Ci/mol) was present throughout the incubation. The [3 H]melatonin in the medium was extracted with chloroform (10). The amount of [3 H]melatonin formed was estimated from the specific activity of the [3 H]tryptophan, a calculation that assumes the specific activity of the melatonin formed from [3 H]tryptophan is the same as that of the tryptophan.

Radioactivity was measured with a Packard Tri-Carb Liquid Scintillation Spectrometer and corrected to absolute values with a Packard Automatic Activity Analyzer. Except where otherwise indicated, statistical analyses were performed by a one-tailed, Mann-Whitney U-test (11). Data are presented as the mean \pm the standard error of the mean.

The following materials were used; [3 H]tryptophan (uniformly labeled, 6.4 Ci/ μ mol, Schwarz BioResearch Co.); [14 C]serotonin creatinine sulfate (56 Ci/mol, Amersham/Searle Co.); serotonin creatinine sulfate and norepinephrine bitartrate (Regis Chemical Co.); acetyl coenzyme A (Mann Research Co.); BGJ^b culture medium (Grand Island Biological Co.).

RESULTS

In vivo studies

Pineal *N*-acetyltransferase activity in normal rats increased markedly during the dark period of a diurnal lighting schedule to about 30-times greater than the activity observed during the light period (Fig. 1). A nearly identical result was observed in sham-operated animals. In contrast to this, ganglionectomy or decentralization of the ganglia resulted in a

nearly total loss of the *N*-acetyltransferase rhythm. In this study, the dark values for these two operated groups were 1.3-1.6 times the light values. These small differences were significant ($P > 0.05$). In addition, after ganglionectomy there was a small increase ($P > 0.01$) in *N*-acetyltransferase activity in the light period in comparison to each of the other groups (normal, sham, and decentralized).

To investigate whether superior cervical ganglionectomy results in a shift in the timing of the *N*-acetyltransferase rhythm, rather than a decrease in its amplitude, operated animals were killed at four different times. Normal animals were killed at the same times to provide a control group. The pineal glands were placed on dry ice after removal, and all four groups were assayed together. The large increase in *N*-acetyltransferase usually observed during a dark period was noted in the normal animals (Fig. 2). The pineals from animals that had been ganglionectomized, however, did not exhibit increased enzyme activity. The minor increase that was observed to occur in ganglionectomized animals at night in the previous experiment was not observed in this study, perhaps as a result of differences in technique.

Organ culture studies

The addition of norepinephrine to pineal organ cultures stimulates *N*-acetyltransferase activity and the formation of labeled melatonin (4, 8, 12, 15). To determine whether the absence of a diurnal rhythm of *N*-acetyltransferase in pineal glands of animals subjected to cervical sympathectomy might be due to changes in the pineal cells themselves, we tested in organ culture pineal glands from animals that had been bilaterally ganglionectomized 3 weeks before death (Fig. 3). All glands were cultured under control conditions for 18 hr, then transferred to fresh medium for the last 6 hr of culture. Half of the pineals were placed in medium that contained norepinephrine. (0.1 mM) during this period. The remaining half were controls. After the 6-hr treatment with norepinephrine, there was an increase both in *N*-acetyltransferase activity in gland homogenates and in the concentration of [3 H]melatonin in the culture medium of the norepinephrine-treated glands, as compared to the controls

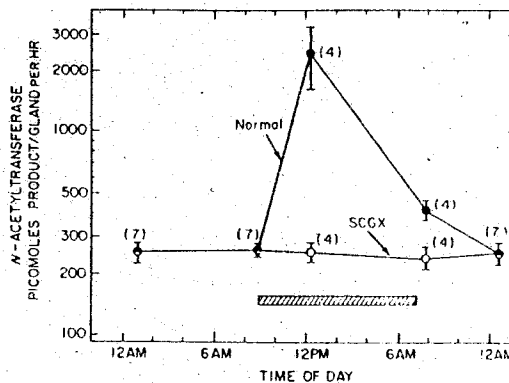


FIG. 2. The effect of removal of superior cervical ganglia on the diurnal rhythm in *N*-acetyltransferase. The numbers in parentheses indicate the number of glands analyzed for each point. The data from normal (●—●) and superior cervical ganglionectomized (SCGX) animals (○—○) were pooled (●—○) where there were no significant difference between means. The shaded bar indicates the duration of the dark period.

(Fig. 3). The response to norepinephrine treatment was nearly identical in chronically denervated glands to that in pineal glands from intact animals. It seemed possible, however, that the response to norepinephrine would only appear in denervated pineal glands after the 18-hr preliminary culture. For this reason, we performed another experiment in which the response of denervated glands to norepinephrine was studied during the first 6 hr of culture (Table 1). The response to norepinephrine treatment in this case was similar to that in the longer-term study in organ culture; both the normal and denervated pineal glands showed about a 10-fold increase in *N*-acetyltransferase activity. These data indicate that chronic denervation does not affect the ability of the pineal cells to respond to norepinephrine.

DISCUSSION

The data presented here demonstrate that the sympathetic innervation of the pineal gland is essential to maintain the 24-hr rhythm of pineal *N*-acetyltransferase activity. The organ-culture studies clearly indicate that denervation does not result in destruction or atrophy of the gland. It would appear that removal or decentralization of the SCG blocks the rhythm by interrupting the flow of neural information from central structures to the pineal gland.

These observations add an important link in the chain of structures and processes known to be involved in the regulation of *N*-acetyltransferase. At one end of this chain is environmental lighting (4). According to recent neurophysiological studies, electrical activity in the rat pineal gland is higher in the dark and is inhibited by light acting via the eye (16). These observations suggest to us that an increase of electrical activity in the pineal gland in the dark stimulates *N*-acetyltransferase activity (see Note Added in Proof).

The mechanism by which light acts is unclear. Perhaps a light-induced electrical signal leaving the eye during the day acts via an inhibitory synapse to block an endogenously generated signal going to the pineal gland. Blockage of the endogenously generated signal would decrease electrical activity in the gland. Only in darkness would this endogenously generated signal reach the gland and stimulate *N*-acetyltransferase. This simple model does not, however, explain the occurrence of a rhythm in *N*-acetyltransferase in blinded animals in constant lighting, or in normal animals in continual darkness (4, 5). Under these conditions, there appears to be endogenous regulation. Perhaps the signal

TABLE 1. *N*-acetyltransferase activity in pineal glands cultured for 6 hr

Treatment group	<i>N</i> -acetyltransferase activity (pmol of ¹⁴ C-labeled product per gland per hr)
Normal controls	121 ± 22.1
+ norepinephrine (0.1 mM, 6 hr)	1580 ± 510*
Chronically denervated controls	127 ± 39.3
+ norepinephrine (0.1 mM, 6 hr)	2429 ± 1070*

Denervated pineal glands were obtained from animals 3 weeks after ganglionectomy. Each group was comprised of four pineal glands.

* Treatment significantly increased the activity of *N*-acetyltransferase relative to control values, $P \geq 0.025$.

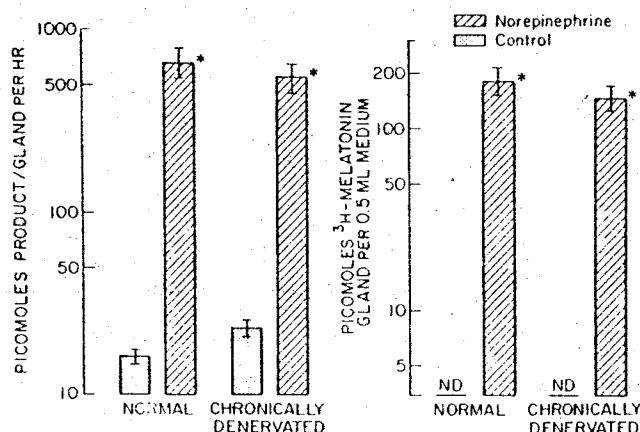


FIG. 3. Pineal organ-culture experiment. Glands for this experiment were obtained from normal animals and animals that had their superior cervical ganglia (SCG) removed 3 weeks before the experiment. Glands were incubated for 24 hr in medium containing [³H]tryptophan. The activity of the pineal *N*-acetyltransferase appears on the left and the amount of [³H]melatonin released into the culture medium during the last 6 hr appears on the right. 0.1 mM Norepinephrine was present only during the last 6 hr. Each group contained three pineal glands that were incubated individually. [³H]melatonin was not detectable (ND) in the medium of control glands.

* Norepinephrine significantly increased this parameter ($P < 0.01$) relative to untreated controls. (2-tailed "t" test).

generator is an oscillator with a period of about 24 hr that only sends a signal to the gland periodically. Or, if this hypothetical oscillator does send a signal continually to the pineal gland, there could be mechanisms in the gland that periodically produce a refractory period during which the gland is insensitive to electrical stimulation. The most probable result of increased electrical activity at nerve endings in the gland is the increased release of norepinephrine. Electrically stimulated discharge of norepinephrine from sympathetic nerve endings has been demonstrated with other tissues (17). The importance of release of neurotransmitter is seen in the results of our studies with animals with decentralized ganglia. The pineal glands in these animals should receive intact innervation from the SCG, but the SCG should receive no input (Fig. 1). Decentralization causes only a 50% decrease in the concentration of norepinephrine in the gland (18). However, in decentralized animals, the nocturnal increase in pineal *N*-acetyltransferase was almost undetectable. Thus, the mere presence of norepinephrine in the pineal gland is not sufficient to establish a rhythm in *N*-acetyltransferase; rather, it appears that this neurotransmitter must be released, presumably in response to increased electrical activity, in order to be effective.

The final link in this transfer of information from the environment to the pineal gland has also been demonstrated in this study. Norepinephrine stimulates the activity of *N*-acetyltransferase (4, 8). This mechanism has been studied in detail and seems to involve the norepinephrine-caused stimulation of pineal adenylate cyclase (19), the elevation of pineal adenosine 3'-5'-cyclic monophosphate production, and the cAMP-caused stimulation of *N*-acetyltransferase via a mechanism that depends upon protein synthesis (8, 20).

What is the physiological importance of the diurnal rhythm in *N*-acetyltransferase? It appears that this enzyme plays a